Mechanisms of *Pantoea agglomerans* Strain E325 as Antagonist of *Erwinia amylovora*

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Abstract

***Pantoea agglomerans* strain E325, the active ingredient in Bloomtime Biological™, was originally isolated from apple blossoms and selected based on broad screening with detached crab apple flowers. To evaluate pH modification on 'Gala' apple stigmas as a possible mode of antagonism, exudates were extracted from flower stigmas and tested with a pH electrode. Measurement of pH in field samples resulted in only slight differences but indicated a low pH range (between 5 and 6) conducive for antibiotic activity based on laboratory assays. An extracellular product of E325 was shown under low-phosphate and low-pH conditions to inhibit *Erwinia amylovora* but not strains of 17 other microbial species tested so far. A minimum of 20 to 40 ng of the compound, purified using RP-HPLC, caused visible inhibition of the pathogen in plate assays. Inhibition was relatively heat stable and unaffected by amino acids, ferric ions or enzymes previously used to characterize antibiotics from other strains of *P. agglomerans*. Conversely, activity was critically affected by pH and phosphate buffering capacity. It was deactivated under basic conditions, and at pH 6 and 7, it diminished or disappeared at a rate that increased with the phosphate buffer concentration. The inhibitory activity was often undetectable at phosphate concentrations commonly used in tests for antibiosis. Work is in progress to develop methodology for direct evaluation of pH on flower stigmas and to further characterize the inhibitory compound produced by strain E325.**

INTRODUCTION

Pantoea agglomerans strain E325, the active ingredient in Bloomtime Biological®, was originally isolated from apple blossoms and selected from more than 1,000 microbial strains evaluated for potential use in fire blight management (Pusey, 1999). In screening assays with detached crab apple blossoms (Pusey, 1997), strain E325 showed exceptional suppressive activity toward *E. amylovora* on stigmatic surfaces and later proved effective in reducing blossom blight on mature apple trees (Pusey, 2002). Like many other microbial strains, E325 is an excellent colonizer of the stigma and likely competes with the pathogen for space and nutrients. Given its high efficacy on flowers, we suspected antibiotic production as another mechanism of antagonism, but detection was elusive. When testing for antibiosis using methods and media of other researchers, inhibition zones either did not appear or their appearance could not be reproduced in assays. To further investigate possible antagonist mechanisms of strain E325 on flower stigmas, stigma exudates from pomaceous flowers were chemically analyzed (Pusey, 2006), and free sugars and amino acids detected in these analyses were incorporated into a partial stigma-based medium (PSBM). The phosphate-buffer concentration was relatively low and suppression of *E. amylovora* by strain E325 in liquid or solid forms of this medium correlated with decreases in pH, as shown in vitro by early workers (Farabee and Lockwood, 1958; Riggle and Klos, 1972). On solid PSBM, inhibition zones also indicated possible antibiosis. The following describes recent efforts to evaluate pH changes on flower stigmas inoculated with strain E325 and to partially characterize an inhibitory product of E325 active under low-phosphate and low-pH conditions.

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MATERIALS AND METHODS

Evaluaton of pH on Inoculated Stigmas

To study the effect of *P. agglomerans* E325 on the pH of blossom stigmas, exudates were extracted from inoculated apple stigmas (*Malus pumila* 'Gala') and evaluated for pH. On three dates in 2005, newly opened flowers were detached and separated into three groups of 50; two groups were inoculated on stigmas with E325 or *E. amylovora* Ea153N (from K. Johnson, Oregon State University, Corvallis) by applying 1–2 µl of suspension (10^8 CFU/ml) per flower, and the third group was not inoculated. After 24 hours at 24°C and 90% relative humidity, stigmas of each of 50 flowers per group were submerged one by one into the same 600 µl volume of water while sonicated for 10 s. After centrifugation and filtration, samples were concentrated to 10X and 50X and pH measured with a pH electrode.

In 2006 and 2007, an orchard experiment with 'Gala' apple involved inoculations on two dates, when flowers were newly opened and 48 h later. Bacterial suspensions were applied with brush to stigmas, using Ea153N at 10^7 CFU/ml and E325 at 10^8 CFU/ml. Treatments were: 1) non-inoculated control, 2) Ea153N applied first date, 3) Ea153N applied second date, 4) E325 applied first date, and 5) E325 applied first date prior to Ea153N on second date. Four or six single-tree replicates were used per treatment. Flowers were periodically sampled to estimate bacterial population sizes and to determine pH of stigma exudates as described.

Isolation and Characterization of Inhibitory Compound

Observation of inhibition zones on solid PSBM were followed by attempts to characterize the active substance. Ingredients of PSBM by weight in 1 L water were: 25 g glucose, 25 g fructose, 0.2 g amino acid mix (proline, asparagine, glutamine and serine in ratio of 3:2:2:1), 0.8 g NH_4Cl, 0.3 g K_2HPO_4, 0.1 g NaH_2PO_4, 0.4 g $MgSO_4$, 1.2 mg nicotinic acid and 15 g agar. Antagonist strain E325 and pathogen strain Ea153N were first grown on nutrient yeast dextrose agar (NYDA) at 24°C for 24 h. A 0.1 ml suspension of E325 (10^8 CFU/ml) from the culture was then spread on PSBM in a single 9 cm plate. After incubation for 24 h at 28°C, growth was removed with a razor blade and agar sections transferred to a 125 ml flask containing 20 ml water. The flask was placed on a rotary shaker at 80 rpm for 1 h and liquid decanted; after repeating twice by adding 20 ml water, decanted liquid was pooled and filter sterilized (~60 ml). The crude filtrate was stored at 5°C and used for characterizing the active compound or for further isolation and purification.

Sensitivity of the inhibitor to heat, pH, phosphate, amino acids and enzymes was tested by treating crude filtrate and transferring 10–12 µl to wells in PSBM. Specific enzymes tested were proteinase K, pepsin, trypsin, α-chymotrypsin, β-lactamase, lipase, bromelain and papain. The medium was overlaid with *E. amylovora* in PSBM agar (100 µl of ~10^9 CFU/ml) and incubated overnight at 28°C. Activity toward other microbial strains was evaluated by streaking cell suspensions across wells to which crude filtrate had been added. Thirty-seven strains were tested, representing 18 species in 10 genera (*Erwinia*, *Pantoea*, *Enterobacter*, *Escherichia*, *Pseudomonas*, *Agrobacterium*, *Bacillus*, *Aureobacterium*, *Micrococcus* and *Geotrichum*).

Purification of Active Compound

Fifty milliliters of crude filtrate was added to a C-18 Plus Sep-pak (Waters, Millford, MA), the column was rinsed with 20 ml water and 20 ml 5% MeOH, then active component eluted with 20 ml 20% MeOH. The active fraction was dried in a rotary evaporator with 50 ml isopropyl alcohol and reconstituted in 500 µl water. Reverse phase high-performance liquid chromatography (RP-HPLC) was employed for purification of the concentrate. Twenty-five µl was injected into an Agilent (Palo Alto, CA) 1100 HPLC coupled with a G1315B diode array detector monitoring at 190–500 nm. Fractions were collected following repeated separations and compiled for further analysis. An Agilent

G1946D mass selective detector (MSD), coupled with atmospheric pressure chemical ionization (APCI) or electrospray-atmospheric pressure ionization (ES-API) source, was adjusted to monitor positive ions and acquire mass spectra ranging from 100 to 2500 *m/z*, and then employed for mass spectral analyses of crude and purified extracts. Dry weight of purified compound was measured and a dilution-end-point of detectible activity determined.

RESULTS AND DISCUSSION

Stigma exudates extracted from 'Gala' apple blossoms showed a reduced pH when inoculated in the laboratory with strain E325, as compared to the non-inoculated control. In contrast, *E. amylovora* increased pH. A similar trend was observed in the field for the third sampling date, and a statistical difference was shown between E325 and other treatments in 2007. Mean pH values for field samples increased over time and were in a narrower range (5.0 to 5.8 overall or within 0.4 pH units on any sampling date) than those of laboratory bioassays (5.0 to 6.8). Mean population sizes of bacteria, as log CFU per flower, varied from 5.5 to 6.3 for strain E325, and from 1.5 to 5.9 for *E. amylovora*, depending on treatment and sampling date. Pre-inoculation of stigmas with strain E325 resulted in reduced levels of *E. amylovora*, as in previous studies (Pusey, 1997; Pusey, 2002).

Since orchard inoculations with strain E325 indicated only slight differences in stigma pH on one late sampling date, results did not implicate pH modification as a primary mode of antagonism toward *E. amylovora*, as proposed by early workers (Farabee and Lockwood, 1958; Riggle and Klos, 1972). The pH values for all field samples, nevertheless, were within a range supporting antibiosis based on later laboratory assays. It is questionable whether pH in extracts from flowers was representative of stigmas and not artifactual, so conclusions about stigma pH may have to await the development and application of more direct methods of pH measurement.

When crude filtrate from PBSM cultures of strain E325 was subjected to various temperatures for 1 h, inhibitory activity toward *E. amylovora* was retained at 105°C, but not at 115°C; after 72 h, activity was retained at 50°C but not 70°C. Adjustment of pH in the filtrate from 3.7 to values from 2 to 11 (by adding 1 M HCl or 1 M NaOH and holding samples at 20°C for 24 h) resulted in inhibition of the pathogen when pH was in the range of 2 to 7 but not 8 to 11. When phosphate in filtrate was increased by concentrations from 2.4 mM to 96 mM, and pH and incubation period were also varied, inhibitory activity remained stable at pH 5. At pH 6, activity diminished after 18 h at phosphate levels from 24 to 96 mM. At pH 7, activity was undetectable after 6 h at 24 to 96 mM phosphate and only slightly detectable after 18 h at 2.4 mM phosphate. Antibiosis was unaffected by amino acids, excess ferric ions and all enzymes tested. Of 37 microbial strains tested so far for sensitivity to the filtrate, only the nine strains of *E. amylovora* tested were sensitive.

Analyses of the purified active substance yielded a single total ion chromatogram (TIC) peak using APCI or ES-API mass spectrometry. The UV-vis signal in the monitored range was weak. Peaks from APCI and ES-API signals displayed distinctive mass spectra with prominent peaks at 556 *m/z* and a dimer peak at 1111 *m/z* (ES-API), suggesting a molecular weight of approximately 555. Dilution-end-point assays indicated inhibitory activity toward *E. amylovora* with a minimum of 20 to 40 ng of the purified compound.

The inhibitory compound has so far exhibited characteristics that distinguish it from other antibiotics of *P. agglomerans* already described. It was unaffected by amino acids or enzymes known to inactivate antibiotics produced by many strains in this group (Ishimaru et al., 1988; Kearns and Hale, 1996; Vanneste, 1996; Vanneste et al., 1992). The E325 compound also was distinguished by inactivity under alkaline conditions or in the presence of phosphate at concentrations generally used in testing for antibiosis. The latter observation explains why inhibitory activity was previously undetected or inconsistent. These findings are congruous with the recent inability to detect *paaB* in

E325 by PCR (Stockwell, unpublished); *paaB* is a gene in the operon for synthesis of pantocin A (Jin et al., 2003), a common antibiotic produced by strains of *P. aggomerans* with efficacy against fire blight. Work is in progress to further characterize the antibiotic of strain E325 and learn more about its possible role in the antagonism of *E. amylovora* on stigmatic surfaces.

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Literature Cited

Farabee, G.J. and Lockwood, J.L. 1958. Inhibition of *Erwinia amylovora* by *Bacterium* sp. isolated from the fire blight cankers. Phytopathology 48:209–211.

Ishimaru, C.A., Klos, E.J. and Brubaber, R.R. 1988. Multiple antibiotic production by *Erwinia herbicola*. Phytopathology 78:746–750.

Jin, M., Wright, S.A.I., Beer, S.V. and Clardy, J. 2003. The biosynthetic gene cluster of pantocin A provides insights into biosynthesis and a tool for screening. Angew. Chem Int. Ed. 42:2902–2905.

Kearns, L.P. and Hale, C.N. 1996. Partial characterization of an inhibitory strain of *Erwinia herbicola* with potential as a biocontrol agent for *Erwinia amylovora*, the fire blight pathogen. J. Appl. Bacteriology 81:369–374.

Pusey, P.L. 1997. Crab apple blossoms as a model for research on biological control of fire blight. Phytopathology 87:1096–1102.

Pusey, P.L. 1999. Laboratory and field trials with selected microorganisms as biocontrol agents for fire blight. Acta Hort. 489:655–661.

Pusey, P.L. 2002. Biological control agents for fire blight of apple compared under conditions limiting natural dispersal. Plant Dis. 86:639–644.

Pusey, P.L. 2006. Biochemical analyses of pomaceous stigma exudates and relevance to biological control of fire blight. Acta Hort. 704:375–377.

Riggle, J.H. and Klos, E.J. 1972: Relationship of *Erwinia herbicola* to *Erwinia amylovora*. Canadian J. Bot. 50:1077–1083.

Vanneste, J.L., Yu, J. and Beer, S.V. 1992. Role of antibiotic production by *Erwinia herbicola* Eh252 in biological control of *Erwinia amylovora*. J. Bacteriology 174:2785–2796.

Vanneste, J.L. 1996. Honey bees and epiphytic bacteria to control fire blight, a bacterial disease of apple and pears. Biocontrol News and Information 17:67N–78N.